MECHANISM OF STARFISH SPAWNING. I. DISTRIBUTION OF ACTIVE SUBSTANCE RESPONSIBLE FOR MATURATION OF OOCYTES AND SHEDDING OF GAMETES[1]

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Since Chaet and his co-workers (Chaet and McConnaughy, 1959; Chaet and Musick, 1960; Chaet, 1964a) discovered that starfish (*Asterias forbesi*) can be induced to spawn by injecting a water extract of radial nerves into the coelomic cavity, some clue to the elucidation of the mechanism of starfish spawning has been afforded. The active substance responsible for gamete-shedding, contained in radial nerves, was reported to be a polypeptide with a relatively small molecular weight (Kanatani and Noumura, 1962, 1964; Chaet, 1964b, 1966). Further, the nerve extract prepared from one species acts similarly in several species, suggesting that the substances are chemically analogous among starfishes (Hartman and Chaet, 1962; Noumura and Kanatani, 1962; Chaet, 1964c, 1966), although there may be some species differences in details; for example, nerve extract of *Asterina pectinifera* is effective in inducing spawning of *Asterias amurensis,* but the converse is not true (Noumura and Kanatani, 1962; Chaet, 1966).

The present investigation was carried out in an attempt to determine the following points with regard to the distribution of active substance in the body of the starfish: (1) Whether or not there exists any difference in the quantity of active substance between male and female starfish, since males seem to shed their gametes more easily than females (Noumura and Kanatani, 1962). (2) Whether or not the active substance is present only in the radial nerves. (3) How the active substance is quantitatively distributed along the radial nerve. (4) Whether the active substance actually appears in the body fluid of the starfish at the time of natural spawning. An attempt is also made to discuss the mechanism of starfish spawning in relation to the results obtained from such investigations.

MATERIALS AND METHODS

The material mainly used was a common Japanese starfish, *Asterias amurensis,* during its spawning season. *Asterina pectinifera* was also used in some experiments dealing with comparative aspects. These starfishes were obtained from Tokyo Bay and kept in crawls hanging in the sea or in aquaria supplied with running sea water at the Misaki Marine Biological Station.

In order to obtain radial nerves, the ambulacral zone was isolated and then perpendicularly, but slightly diagonally, cut with scissors into two halves along the midline so that the nerve could easily be observed. These nerves were stripped off with fine forceps and collectd. The nerves were stored frozen and dried by lyophilization. This method for obtaining the radial nerves seems to be more simple than the original method of Chaet (1964a).

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For obtaining the nerve ring, a starfish was placed oral side up in a large Petri dish containing sea water, and the oral spines and tube feet near the mouth were carefully removed under a dissection microscope (Fig. 1). The nerve ring thus easily observed was cut out with fine scissors and lyophilized.

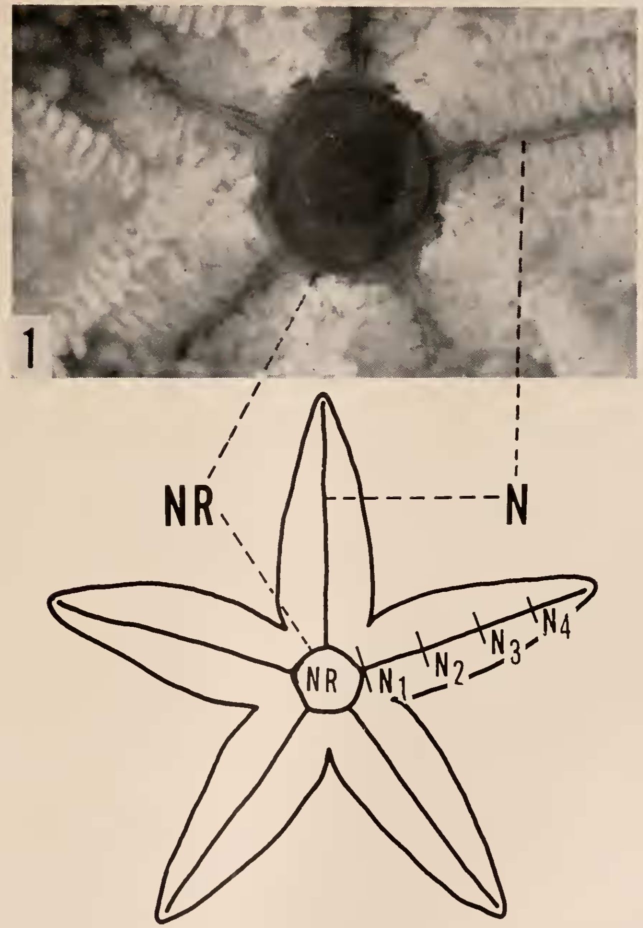

FIGURE 1. Nerve ring (NR) and radial nerve (N) of *Asterias amurensis.* Lower illustration shows parts of nerve used in experiment.

In an experiment comparing the activities of the various parts of radial nerves, the ambulacral plate was cut, after removing the nerve ring, into four sections as shown in Figure 1, and then the radial nerve of each part was stripped off as described above and separately lyophilized. The parts of the radial nerves were designated as N_1, N_2, N_3, and N_4, from proximal to distal.

To make the nerve extract, a few milliliters of cold de-ionized water were added to the lyophilized material, which was then homogenized. To the homogenate, de-ionized water was added to give a concentration of 4 mg./ml. of lyophilized nerve and the mixture was centrifuged for one hour at 20,000 *g*. An equal volume of 1 *M* sodium chloride was added to the supernatant to make an isotonic nerve extract, which served as the original extract. In preparing test solutions, the original extract was diluted at various concentrations with sea water (successively diluted twice from 100 μg./ml. to 0.8 μg./ml., or from 200 μg./ml. to 1.2 μg./ml.). An extract of tube feet was made by the same procedure: the concentrations of the test solutions were adjusted from 500 μg./ml. of lyophilized tube feet to 10 μg./ml. by serial dilution with sea water. Extracts of body wall (ectoderm of the aboral surface of the arm), cardiac stomach and pyloric caeca were made by homogenizing the lyophilized samples in sea water. (The body wall was stripped off with fine forceps.) The homogenates were heated in a boiling water bath for five minutes and centrifuged. The supernatants were serially diluted with sea water. The concentrations with adjusted at 1000-32 μg./ml. for body wall, 5000-312 μg./ml. for cardiac stomach and 50-1.57 mg./ml. for pyloric caeca.

To assay the capacity of the test solutions to cause spawning and meiosis, an isolated small fragment of ovary, about 10 to 12 mm. in length, was placed in a small Petri dish containing 4 ml. of the solution and observed (Fig. 2) (*cf.* Chaet. Andrews and Smith, 1964). Ovarian fragments derived from a single female were used in each experiment in order to eliminate individual differences in reactivity. These ovarian fragments had previously been rinsed in sea water for an appropriate period until no more eggs were released from their cut surfaces. *In vivo* assays were also performed by injecting the test solution into the coelomic cavity of intact starfish.

To obtain coelomic fluid, a small slit was made in the aboral side of the distal part of an arm and the coelomic fluid was collected through it. The coelomic fluid thus obtained was used immediately after centrifugation for 30 minutes at 4000 r.p.m., or stored in a deep freezer before use.

RESULTS

Comparison of content of active substance between males and females

The radial nerves taken from each of four male and four female *Asterias* (arm length 10 cm.) were separately homogenized in 3 ml. of de-ionized water. The homogenate was diluted with an equal volume of 1 *M* sodium chloride, and 0.5 ml. of this nerve extract was injected into each of five starfish. As shown in Table I, the nerve extract obtained from male starfish was effective in inducing spawning of females, and *vice versa*. After shedding the gametes, the gonads were very small in most of these animals. Isolated ovaries placed in 50 ml. of sea water containing 2 ml. of these nerve extracts began to shed simultaneously (after about 30 minutes), regardless of the sex of the nerve donors. Control ovaries, placed in sea water containing 2 ml. of 0.5 *M* sodium chloride, failed to spawn. These preliminary experiments suggested that the spawning factor is the same in the two sexes.

Nerve extracts prepared from males were also effective in inducing meiosis of oocytes.

Experiments were next conducted to determine whether there exists any difference between male and female *Asterias* with respect to the content of shedding substance in the radial nerves, since the males shed in response to lower concentrations of injected nerve extract than do the females. Small fragments of the ovaries taken from a single female were exclusively used for assay in each experiment.

TABLE I

Induction of spawning by injected nerve extracts obtained from male and female Asterias

Groups of experiments	Nerve extracts obtained from					
	Male			Female		
	Sex	Time (min.)*	Amount of** gametes	Sex	Time (min.)	Amount of gametes
1	Male	29	+++	Female	39	+++
	Female	51	++	Female	39	+++
	Male	34	+++	Female	43	++
	Female	51	+++	Female	40	+++
	Male	34	++	Male	33	++
2	Male	26	+++	Male	26	+++
	Male	27	+++	Female	30	+++
	Male	28	+++	Female	32	+++
	Male	32	+++	Male	35	+++
	Female	40	++	Male	34	+++
3	Female	46	+	Male	28	++
	Male	33	++	Male	34	+++
	Male	No spawning***	—	Female	29	++
	Male	40	++	Female	37	+++
	Female	40	++	Female	37	++
4	Male	29	+++	Male	29	+++
	Female	35	+++	Male	29	+++
	Male	36	+++	Female	32	+++
	Female	No spawning***	—	Female	49	+
	Male	32	+++	Female	51	+++

* Interval preceding discharge of gametes.

** +++: large amount of gametes; ++: intermediate amount of gametes; +: small amount of gametes.

*** Gonads poorly developed, or had already spawned.

These ovarian fragments were placed in test solutions containing the original nerve extract at various concentrations (0.8–50 μg. of lyophilized nerve per ml.), prepared separately from males and from females, and their spawning reactions were examined after one hour. Table II shows the results of six pairs of such experiments. These data clearly demonstrate that the content of shedding substance in radial nerves is the same in the two sexes.

TABLE II

In vitro assay to test for sex difference in gamete-shedding activity of nerve extracts. Ovary fragments derived from one female were used in each pair of experiments

Concentration of nerve extracts (μg./ml. lyophilized nerve)	Comparison of shedding induced by nerve extracts obtained from ♂ and ♀ donor starfish. A-F: ovarian fragments from 6 different females.											
	A		B		C		D		E		F	
	m.	f.*	m.	f.	m.	f.	m.	f.	m.	f.	m.	f.
50	+	+	+	+	+	+	+	+	+	−	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+
12.5	+	+	+	+	+	+	+	+	+	+	+	+
6.3	+	+	+	+	+	+	+	+	+	+	+	+
3.1	−	−	−	−	+	+	+	+	−	−	+	+
1.6	−	−	−	−	+	+	−	−	−	−	−	−
0.8	−	−	−	−	−	−	−	−	−	−	−	−

+: positive, −: negative.
* Sex of nerve donor.

Quantitative distribution of active substance in various parts of radial nerve and in nerve ring

The results of *in vitro* assay of the active substance contained in the nerve ring and various regions of the radial nerves (Fig. 1) are shown in Table III. These data indicate that the active substance is present in the nerve ring as well as in the radial nerves, and is evenly distributed in quantity per dry weight among the nerve ring and the various parts of the radial nerves.

The absolute amount of active substance thus decreases from proximal to distal as the size of the cross-section of the radial nerve decreases (Table IV). The ratio of weights of lyophilized nerve at the various regions thus reflects the actual content of the active substance along the radial nerve.

In these *in vitro* experiments, the ovarian fragments began to shed after about 20–25 minutes (in some cases less than 20 minutes) when they were immersed in test solutions (4 ml.) containing more than 25 μg./ml. of the lyophilized nerve.

TABLE III

Distribution of the gamete-shedding substance in nerve ring and radial nerves (Fig. 1) of Asterias

Minimum dose of nerve extract for induction of spawning within 1 hour in ovary fragments (μg./ml. lyophilized nerve)						
Nerve donors	A(♂)	B(♂)	C(♂)	D(♀)	E(♀)	F(♀)
Parts of nerves						
NR	12.5	6.3	6.3	6.3	6.3	3.1, 6.3*
N_1	12.5	6.3	6.3	6.3	6.3	3.1, 6.3*
N_2	12.5	12.5	6.3	6.3	6.3	3.1, 6.3*
N_3	6.3	6.3	6.3	6.3	6.3	3.1, 6.3*
N_4	6.3	12.5	3.1	6.3	6.3	3.1, 3.1*

* Ovary from different animal.

In *in vivo* experiments, female starfish injected with 1 ml. of 0.5 *M* sodium chloride containing 500 μg. of the same lyophilized nerve material usually began spawning after about 40 minutes.

Similar *in vitro* experiments on *Asterina pectinifera* clearly confirmed the above results obtained in *Asterias;* the minimum dose for induction of spawning was the same among the various regions of radial nerves and nerve ring (10 μg./ml. of lyophilized nerve in the middle of June and 2.5 μg./ml. in the end of June, respectively).

Presence of active substance in body parts other than central nervous system

Since nervous tissue is plentiful in the tube feet (Smith, 1937), the gamete-shedding activity of a tube-foot extract was next examined in a similar way. Table V shows the results of an *in vitro* assay, one hour after ovarian fragments were immersed in tube-foot extracts. These data show that the tube feet also contain shedding substance, and that there is little difference as regards its content between males and females. The minimum doses for induction of spawning were 50 to 100 μg. of lyophilized tube feet per ml., when the experiments were conducted later in the spawning season. When such experiments were made earlier in the season, the minimum doses for induction of spawning ranged between 150 and 200 μg./ml. Furthermore, breakdown of germinal vesicles had begun in most of the eggs discharged from ovarian fragments 30 minutes after immersion in test solutions containing 200 μg. of lyophilized tube feet per ml. These data indicate that the tube feet actually contain the active substance, although the amount is small as compared with that in the radial nerves.

Table IV

Weights of various parts of lyophilized Asterias nerves (Fig. 1)

Region	NR	N_1	N_2	N_3	N_4
Weight* (mg.)	2.0 ± 0.1**	9.0 ± 0.9	8.3 ± 1.5	6.7 ± 0.7	3.6 ± 0.3
Ratio	1	4.5	4.2	3.4	1.8

* Average of six animals (*ca.* 11 cm. arm length) shown in Table III.
** Standard deviation.

Some additional experiments were also carried out to determine whether the active substance exists in body regions other than the central nervous system and the tube feet. When the gamete-shedding activity of the body wall was tested *in vitro* with ovarian fragments, it was found that 125 μg./ml. or more of the lyophilized material were effective. The extract of the cardiac stomach also showed shedding activity; 625 μg./ml. or more were effective, whereas the extract of pyloric caeca had no effect even at a concentration of 25 mg./ml.

Occurrence of active substance in coelomic fluid at time of natural spawning

The injection of coelomic fluid, taken from an animal (*Asterias*) before or after spawning, into the coelomic cavity of another starfish with ripe gonads, failed to induce spawning.

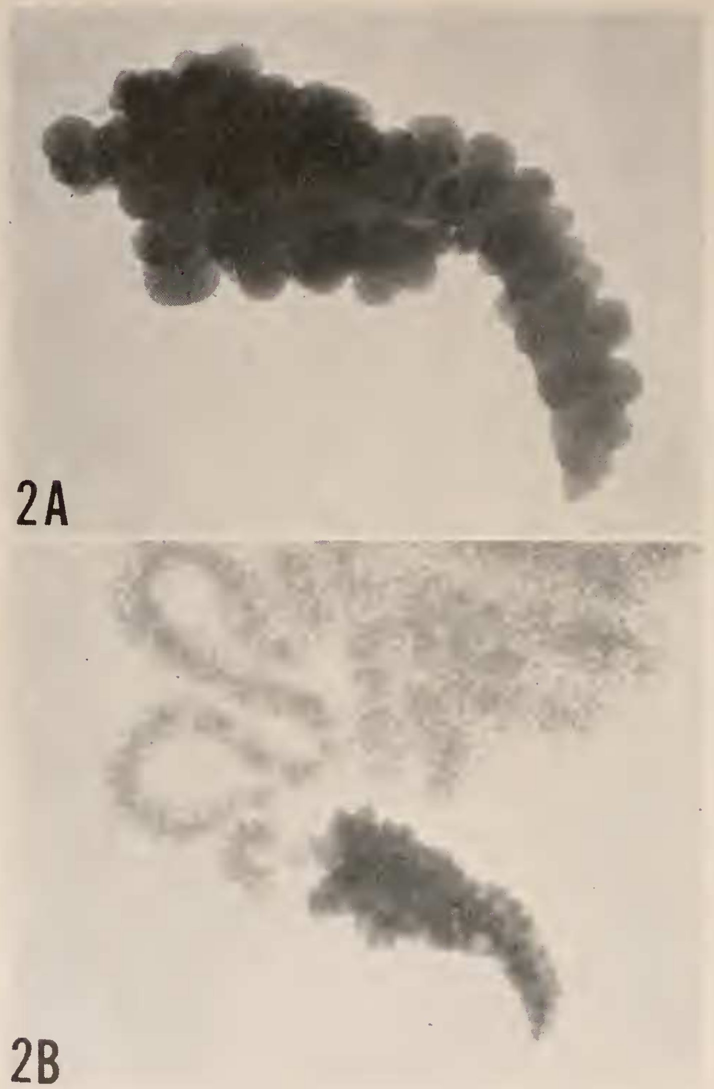

FIGURE 2. *In vitro* assay of gamete-shedding substance. A: ovarian fragment (*ca.* 12 mm.) of *Asterias amurensis.* B: same ovarian fragment discharging eggs after treatment with nerve extract. Same magnification.

However, coelomic fluid obtained from starfish at time of spawning showed gamete-shedding activity. When coelomic fluid was collected from a starfish spawning naturally in the laboratory and at once injected *in toto* into another starfish, spawning occurred in some cases.

In vitro experiments were next performed in order to verify more clearly the presence of the shedding substance in the coelomic fluid of naturally spawning starfish. Five to 20 ml. of coelomic fluid were separately collected from each of six spawning animals (three males and three females). Small ovarian fragments were separately exposed to 2.5 ml. of such coelomic fluid. Controls were exposed to coelomic fluid from six other starfish which were not spawning.

The ovarian fragments immersed in the control coelomic fluid did not shed their eggs and the germinal vesicles of the ovarian eggs remained intact. On the contrary, spawning was induced within 15 to 20 minutes in all of the experimental fragments. The germinal vesicles in all the discharged eggs disappeared within 30 minutes after exposure of the fragments to the coelomic fluid. Observations after one hour revealed strongly shrunken ovarian fragments with thick alveolar walls, containing few, if any, eggs. These observations clearly indicate that the active substance is actually present in the coelomic fluid of a spawning starfish. When these test fluids were filtered and ovarian fragments taken from another starfish were immersed in them, shedding began after 14 to 27 minutes and the germinal vesicles underwent breakdown within 30 minutes. The degree of shedding reaction in these cases corresponded to that of the ovarian fragments exposed to the radial nerve extract containing 12.5 μg./ml. or more of lyophilized material. However, after the use of the same coelomic fluid twice in this way, the shedding activity decreased to some extent: ovary fragments in some experimental lots which were immersed for the third time began to shed only after 30 minutes and the spawning was incomplete. Germinal vesicles remained intact in some of the eggs within the ovary (10–20%). The shedding reaction in the third case seems to correspond to the case in which about 6 μg./ml. of lyophilized radial nerve were used. This reduction in inducing capacity suggests that the active substance is in some way taken up by the ovarian fragments, or that some inhibitory substance is released into the experimental coelomic fluid from the ovarian fragments.

Table V

In vitro assay of the gamete-shedding substance contained in tube feet of Asterias

Minimum amount of tube-foot extract necessary to induce spawning within 1 hour in ovarian fragments from 4 females									
Tube feet from males Exp. No.	μg./ml. lyophilized tube feet				Tube feet from females Exp. No.	μg./ml. lyophilized tube feet			
1	100,	100,	100,	50,	1	100,	100,	50,	100,
2	100	100	100	100	2	100	100	100	50
3	100	100	150	—	3	100	100	100	100
4	100	100	100	100	4	150	100	50	50
5	50	100	50	50	5	50	100	50	50
6	50	100	100	—	6	50	100	50	—

Discussion

According to Unger (1962), a substance which causes shedding of sperm is extractable from the radial nerves of male starfish only, in *Asterias glacialis*. The results of the present study, however, clearly demonstrate the presence of sperm-shedding substance in the radial nerves of female as well as of male *Asterias* *amurensis* (Table I). The so-called egg-shedding substance was also obtained from male starfish; moreover, *in vitro* experiments using isolated ovary fragments showed that the content of the active substance in the radial nerves is equal in the two sexes. Therefore, the substance responsible for inducing the release of sperm is believed to be identical with the substance which induces the release of eggs. The active substance was also found to be equally effective in inducing spawning in both sexes at several steps in the course of chemical purification, and could not be separated into two different substances specific to males or females (Kanatani and Noumura, 1964; Kanatani, unpublished). Chaet (1966) has recently reported that the shedding substance is present in *Asterias forbesi* nerves of both sexes, and in the same concentration. According to his experiments using intact starfish, shedding substance taken from nerves of either sex stimulated shedding in both sexes.

With respect to the location of the gamete-shedding substance in a single starfish, Chaet (1966) reports that hot (76° C.) salt water extracts prepared from various tissues other than radial nerve of *Asterias forbesi,* including tube feet and the oral and aboral surfaces, show no gamete-shedding activity. It is quite possible that his extraction procedure was inadequate in this case. As is clearly shown in our present results, the gamete-shedding substance is not confined to the nerve ring and radial nerve. For example, extracts of tube feet, body wall and cardiac stomach also showed the shedding activity. Considering the order of the shedding activity expressed by the extracts of various regions of the body on the one hand, and the results of the extensive study of Smith (1937) on the distribution of the nervous system of starfish on the other hand, the quantitative distribution of the active substance in the starfish seems to correspond to the quantity of nervous tissue present in a given part of the body. Within the radial nerve, Uter (1966) has reported that the active gamete-shedding substance is located only in the most aboral region of the nerve.

Chaet (1964c, 1966) has suggested that the radial nerves also contain a substance, "shedhibin," which inhibits the action of the gamete-shedding substance. According to his opinion, natural control of shedding is regulated both by the level of shedding substance and the presence (or absence) of shedhibin. The nature of shedhibin, however, is still obscure. In this connection, attention must be drawn to the observation made in the present study, that the gamete-shedding substance is present in the coelomic fluid only at the time of spawning, even during the breeding season. That the shedding substance can act directly on the gonads has been clearly demonstrated in our previous work, in which the gamete-shedding substance was locally applied to an isolated ovary (Kanatani, 1964). The ripe, distended gonads, therefore, will readily respond to the shedding substance when it is released into the coelomic cavity. It thus appears highly probably that spawning is controlled by some mechanism which introduces the gamete-shedding substance into the coelomic cavity in which the gonads are suspended. The problems remaining to be solved are: (1) from what part of the nervous system and through what route the shedding substance reaches the coelomic cavity; and (2) by what means such transport of the substance is controlled.

It is unfortunately difficult to determine the actual amount of gamete-shedding substance released into the coelomic cavity, because the substance, when released into the cavity, seems to be readily absorbed by the gonads which occupy most of the space and present an extremely large surface area because of their complicated branching structure. *In vitro* experiments on the shedding activity of coelomic fluid carried out in the present study strongly suggest that the substance is actually taken up by the ovarian fragments. Evidence that *Asterias* gonads can absorb labeled amino acids and glucose from dilute solution in sea water and coelomic fluid (Ferguson, 1964b) supports this suggestion.

In the foregoing considerations the gamete-shedding substance is thought of as being transported first into the coelomic cavity and acting directly, from the outside, upon the gonads. However, it is also possible that the shedding substance responsible for natural spawning is released directly into the gonad, either from the genital sinus *via* the aboral coelomic sinus or from the nerves distributed in the gonad, with an excess of the substance diffusing into the coelomic fluid through the coelomic epithelium. Against this possibility, histological observation of the ripe, distended ovary shows that the genital sinus is so strongly compressed as to be hardly discernible. Moreover, the strictly localized effect of externally applied nerve extract (Kanatani, 1964) argues against the existence of an internal transport system within the ovary. Finally, the gonads are less well innervated than other organs. On the other hand, shrinkage of the gonads takes place in the same way after natural spawning as after spawning induced by the presence of nerve extract in the coelomic cavity. Moreover, the recent investigations of Ferguson (1964a, 1964b) have demonstrated that the starfish coelomic fluid plays an important role in the transport and exchange of some substances between tissues, regardless of whether they are located in the coelomic cavity or in some other space.

It is therefore considered highly probable that at the time of spawning the shedding substance is released into the coelomic fluid and taken up by the gonads, where it acts to bring about meiosis of oocytes and spawning of the gametes.

Although it is known that the gonad becomes more sensitive toward the end of the spawning season, it has not yet been determined whether the shedding substance is abruptly released into the coelomic cavity at each spawning or accumulates little by little until a threshold concentration is reached.

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SUMMARY

1. The localization in the starfish body of the active substance responsible for maturation and gamete-shedding was determined by *in vitro* assay, using *Asterias amurensis* and *Asterina pectinifera*.

2. The active substance was found in the radial nerves at the same concentration in both male and female starfish, suggesting that the testis responds more readily to the action of the substance than does the ovary. The gamete-shedding substance seems to be identical in the two sexes.

3. As determined by the shedding reaction of isolated fragments of ovaries, the quantity of active substance (per dry weight of lyophilized nerve materials) was found to be uniform in various parts of the nerve ring-radial nerve system.

4. Tube feet and the body wall also contained considerable amounts of the active substance, although the content was several times lower than that of the radial nerves.

5. The shedding activity was also detectable in the extracts of some other regions of the starfish in which nervous tissue is plentiful: for example, cardiac stomach. However, the activity was much less than that of the radial nerves.

6. The active substance was found in the coelomic fluid only when the starfish were undergoing natural spawning. The coelomic fluid of starfish which were not spawning did not show any shedding activity, regardless of the condition of their gonads.

7. The significance of the appearance of active substance in the coelomic fluid in relation to the mechanism of starfish spawning is discussed.

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